



ORIGINAL ARTICLE

Aflatoxin production and in vitro toxicity of *Aspergilli* section *Flavi* isolated from air samples collected from different environments

Daniela Jakšić¹ · Sándor Kocsubé² · Ottó Bencsik² · Anita Kecskeméti² · András Szekeres² · Dubravko Jelić³ · Nevenka Kopjar⁴ · Csaba Vágvolgyi² · János Varga² · Maja Šegvić Klarić¹

Received: 14 August 2018 / Revised: 4 February 2019 / Accepted: 22 February 2019 / Published online: 12 March 2019

© Society for Mycotoxin (Research Gesellschaft für Mykotoxinforschung e.V.) and Springer-Verlag GmbH Germany, part of Springer Nature 2019

Abstract

Aspergilli section *Flavi*, originally isolated from air samples collected from inhabited apartments (AP), unoccupied basements (BS), and processing facilities of a grain mill (GM), were analyzed for their potential to produce aflatoxin B₁ (AFB₁) on solid media. The isolates were further characterized with regard to their cytotoxic, genotoxic, and pro-inflammatory properties in vitro. *Aspergilli* were identified based on partial calmodulin (*CaM*) gene sequencing; the producing capacities of isolates were analyzed by HPLC/FLD and confirmed by genes in biosynthesis (*aflR*, *norA*, *omtA*). In the grain mill, the *Aspergilli* section *Flavi* (up to 1.3×10^6 cfu/m³) dominated by AFB₁-producing *Aspergillus flavus* (71%, 4.5–5254 ng/ml) which showed a serious health risk for workers. Living environments were not relevant sources of exposure. After 24 h, AFB₁ (1–100 µmol/l) reduced cell viability (MTT test) in both A549 cells and THP-1 macrophage-like cells without reaching IC₅₀. In A549 cells, the extract of the AFB₁-producing *A. flavus* significantly decreased cell viability but not below 50%. THP-1 macrophage-like cells were more sensitive to both extracts, but IC₅₀ was obtained only for the AFB₁-producing strain (0.37 mg/ml; AFB₁ 2.78 µmol/l). AFB₁ (1 and 10 µmol/l) induced significant DNA damage (tail intensity, alkaline comet assay) in A549 cells in contrast to *Aspergilli* extracts. AFB₁ elevated IL-6 and IL-8, while *Aspergilli* extracts increased IL-1β, TNF-α, and IL-17 release in THP-1 macrophages (ELISA). Chronic exposure to AFB₁ and/or other metabolites in airborne *A. flavus* from occupational environments may stimulate epithelial damage of airways accompanied by lowered macrophage viability.

Keywords Airborne fungi · Aflatoxin B₁ · Cytotoxicity · DNA damage · Cytokines

Introduction

The *Aspergillus* section *Flavi* comprises several species widely distributed in the environment. These species can be separated into two groups based on their economic importance and impact on human and animal health. The first group includes the non-producing aflatoxin species *Aspergillus oryzae*,

Aspergillus sojae, and *Aspergillus tamarii* used in oriental food fermentation (Campbell-Platt 1994). Genetically modified *A. oryzae* strains are used for the production of enzymes, including lactase, pectin esterase, lipase, protease, and xylanase (Pariza and Johnson 2001). The second group includes aflatoxin-producing species *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*, which contaminate and damage various pre-harvested and stored agricultural products (Perrone et al. 2014). In the past decade, the production of aflatoxins was also reported in newly described species *Aspergillus arachidicola*, *Aspergillus bombycis*, *Aspergillus minisclerotigenes*, *Aspergillus parvisclerotigenus*, *Aspergillus pseudocaelatus*, *Aspergillus pseudonomius*, *Aspergillus pseudotamarii*, *Aspergillus togoensis*, *Aspergillus mottae*, *Aspergillus sergii*, and *Aspergillus transmontanensis* (Varga et al. 2011; Soares et al. 2012).

Aflatoxins are known hepatotoxic mycotoxins with carcinogenic, genotoxic, and teratogenic properties in both humans and animals (Bennett et al. 2003). Among these mycotoxins,

✉ Maja Šegvić Klarić
msegi@pharma.hr¹ Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Schrottova 39, 10000 Zagreb, Croatia² Department of Microbiology, Faculty of Science and Informatics, University of Szeged, H-6726, Szeged Közép fasor 52, Hungary³ Fidelita Ltd., Prilaz baruna Filipovića 29, 10000 Zagreb, Croatia⁴ Mutagenesis Unit, Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10000 Zagreb, Croatia

aflatoxin B₁ (AFB₁) is the most toxic as well as the most frequently occurring aflatoxin in agricultural products (Varga et al. 2011).

In general, aflatoxins are principally produced by *A. flavus* and *A. parasiticus*; *A. flavus* mainly produces AFB₁ and AFB₂, while most *A. parasiticus* isolates produce AFG₁ and AFG₂ in addition to AFB₁ and AFB₂. However, *A. flavus* is considered more invasive and out-competes *A. parasiticus* when both species are present in soil (Perrone et al. 2014). Aflatoxigenic strains of *A. flavus* are most widespread in tropical and subtropical areas. However, in recent years, a more frequent occurrence of aflatoxin producers has been detected in temperate-climate regions, associated with current climate change. Air humidity changes (abundant rain, droughts), increase in temperature, and increased CO₂ concentration directly affect the expression of regulatory (*aflR*) and structural genes (*aflD*) involved in the biosynthesis of AFB₁ and, thus, correlate with its biosynthesis (Paterson and Lima 2010; Medina et al. 2014).

Next to *A. fumigatus*, *A. flavus* is the second most frequent cause of invasive and non-invasive aspergillosis in humans; it may also cause sinusitis, cutaneous and wound aspergillosis, as well as otitis and keratitis (Hedayati et al. 2007; Manikandan et al. 2013).

Taking into account the aflatoxigenic ability of the listed Aspergilli section *Flavi*, particularly *A. flavus*, as well as its role in the etiology of several diseases in humans, an accurate identification of the species would provide fundamental information concerning their aflatoxigenic and pathogenic properties (Godet and Munaut 2010). Several molecular genetic techniques have been developed to distinguish the *Aspergillus* species in the section *Flavi*; Varga et al. (2011) proposed a polyphasic approach, including morphological characters, extrolite data, and partial calmodulin (*CaM*), β -tubulin (*BenA*), and ITS sequences as well as the presence of genes involved in aflatoxin biosynthesis.

Since the discovery of aflatoxins, the hepatotoxic action of AFB₁ has been mainly studied in various experimental models through AFB₁-contaminated food consumption (Bennett et al. 2003). However, a link between AFB₁ inhalation in an industrial setting and liver or lung cancer incidence and mortality has been suggested by several investigations (Olsen et al. 1988; Viegas et al. 2012). Taking into account the lack of data on human exposure to fungal burden in urban homes and occupational settings in our country, recently, we conducted a year-round investigation of airborne fungi in living (apartments, basements) and occupational (grain mill) indoor environments in Croatia with special attention to *Aspergillus* sections *Flavi*, *Nigri*, and *Versicolores* due to their known pathogenic and/or mycotoxin-producing properties (Jakšić Despot and Šegvić Klarić 2014,b). Based on morphological methods, Aspergilli sections *Flavi* and *Nigri* were recovered in the highest frequencies (50–100% and 15–55%, respectively)

from samples taken in the grain mill, while Aspergilli section *Versicolores* was more abundant in apartments and basements (10–65%) (Jakšić Despot and Šegvić Klarić 2014,b). Before appropriate molecular identification methods for species assigned to these sections, indoor air isolates were usually reported as *A. flavus*, *A. niger*, or *A. versicolor* (Samson et al. 2014). As we previously noted, accurate identification of the *Aspergillus* species is fundamental with regard to their toxigenic and/or pathogenic properties (Godet and Munaut 2010). Thus, herein, we described the species diversity of *Aspergillus* sections *Nigri* (Jakšić et al. 2018) and *Versicolores* (Jakšić Despot et al. 2016) using calmodulin sequence-based methods, their ability to produce fumonisin B₂ and sterigmatocystin, respectively, and to explore toxic potential of Aspergilli extracts in comparison with these mycotoxins alone. To further extend knowledge on *Aspergillus* section *Flavi* biology and toxicology, the present study aimed to explore the species diversity of isolated strains using a polyphasic approach (morphological methods, *CaM* sequence, and presence of genes taking part in aflatoxin biosynthesis) and confirm aflatoxin-producing abilities in Aspergilli extracts by HPLC/FLD.

Some studies showed that AFB₁ alone induces several toxic events in human airways, including damage of airway epithelial cells, decrease of the ciliary beat frequency (Lee et al. 2016), DNA damage due to formation of exo-AFB₁-8,9-epoxide by human lung microsomes (Kelly et al. 1997) as well as takes a part in inflammation (Lee et al. 2016; Luongo et al. 2014). However, the effects of inhaled aflatoxin-producing fungi on the airway epithelium have not been well-characterized so far.

As *A. flavus* was among the most abundant species in the grain mill, it was justified to explore the cytotoxic, genotoxic, and immunomodulatory properties of AFB₁-producing and AFB₁-non-producing *A. flavus* extracts in contrast to AFB₁ alone in order to clarify whether particular toxic properties of Aspergilli could be attributed to AFB₁. This was carried out using human lung adenocarcinoma A549 cells and macrophages derived from human leukemic monocyte THP-1 cells as experimental models.

Materials and methods

Chemicals and media

Methanol, acetonitrile, methylene chloride, ethyl acetate, and n-hexane for sample preparation and eluents were purchased from VWR (Debrecen, Hungary). Formic acid, trifluoroacetic acid, and aflatoxin B₁ standard were obtained from Sigma-Aldrich (Budapest, Hungary). Deionized water for both sample preparation and high-performance liquid chromatography (HPLC) runs was produced by Merck Millipore Milli-Q

Gradient A10 water purification equipment (Budapest, Hungary). Dichloran 18% glycerol agar (DG-18), Czapek Yeast Agar (CYA), and Malt Extract Agar (MEA) were purchased from Oxoid (Hampshire, UK). Media for cell culture maintenance, including RPMI 1640, fetal bovine serum (FBS), trypsin-EDTA, phosphate-buffered saline (PBS; Ca^{2+} and Mg^{2+} free), penicillin, and streptomycin were from Lonza (Basel, Switzerland). MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], phorbol 12-myristate 13-acetate (PMA) ethidium bromide, acridine orange, normal melting point (NMP) agarose, low melting point (LMP) agarose, Triton X-100, Tris buffer, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Deisenhofen, Germany). Dimethyl sulfoxide (DMSO) ethanol HCl, NaCl, Na_2EDTA , and NaOH were from Kemika (Zagreb, Croatia).

Sampling areas

The samples of airborne fungi were collected over a one-year period (2012) in two-month intervals at the processing facilities of a grain mill (GM) situated near Zagreb, Croatia, as well as in residential locations in the city which included two inhabited apartments (AP) and two unoccupied basements (BS) as well as outdoor air (ODA) as described in detail in Jakšić Despot and Šegvić Klarić (2014,b). In each period of sampling in GM, twenty samples were collected during milling operations at the site of grain/flour exchange, site of flour storage, site of sieving, and site of milling. In each AP (approximately 70 m²), samples were taken at five locations, including the kitchen, dining room, living room, bedroom, and bathroom, in duplicate. BSs were located in the same buildings as the AP, and residents used these spaces to store various items (e.g., wooden, metal, plastic, or glass materials). In each BS, samples were taken at five locations in duplicate. Samples of outdoor air were taken in proximity to indoor locations, and, during each sampling period, ten ODA samples were collected.

The sampling approach included collecting 20 indoor samples and 10 outdoor samples per each location and at a given time point. Altogether, 420 individual samples were taken.

Sample collection

Airborne fungi were sampled using an Airsampler Mas-100 Eco (Merck, Berlin, Germany) with 400 holes (hole to agar impactor, impaction velocity 10.8 m/s, and airflow rate 100 l/min) and DG-18 agar plates (Samson et al. 2010). Because of the high contamination level, a volume of 10 l was chosen for sampling in the GM, while a volume of 50 l was applied at the other sampling locations. The plates were incubated at 25 °C \pm 0.2 °C in the dark for five days, after which the developed fungal colonies were counted, and results were expressed as colony-forming units per cubic meter (cfu/m³). Aspergilli

were isolated on CYA and MEA agar plates (Samson et al. 2010). Isolates assigned to the *Aspergillus* section *Flavi* were purified and cultivated on CYA and MEA plates at 25 °C in the dark for seven days. Morphological identifications were carried out according to literature (Samson et al. 2010).

Determination of Aspergilli section *Flavi* genotypes

Isolation of genomic DNA from mycelia grown in liquid YPD medium (1% Bacto yeast extract, 1% Bacto peptone, 1% D-glucose) for five days was performed by Masterpure™ yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. The isolated DNA was diluted to < 1.000 ng and used as template DNA in PCR reactions. A fragment of the calmodulin gene (*CaM*), part of the transcriptional regulator of aflatoxin biosynthesis (*aflR*), norsolorinic acid reductase (*norA* or *aflE*), and O-methyltransferase gene (*omtA*, *aflP*) (Yu et al. 2004) were amplified using the primers specified in Table 1. Each PCR reaction mixture (20 µl) contained 0.2 mmol/l deoxyribonucleotide triphosphate (dNTP), 0.2 µmol of primers, 1 U DreamTaq DNA Polymerase (Thermo Scientific, Madison, WI, USA) with the respective buffer (with 1.5 mmol/l MgCl_2) (Thermo Scientific, Madison, WI, USA), nuclease-free water, and template DNA. Amplifications were performed using a Thermocycler T-100 (BioRad, Budapest, Hungary). The PCR cycling protocol consisted of 35 cycles, including an initial denaturation at 95 °C for 2 min in the first and 20 s in the following runs, annealing temperature as specified in Table 1 for 20 s, elongation at 72 °C for 40 s, and 2 min for the final elongation. The amplification products were analyzed by electrophoresis on 2% agarose gels using fluorescent dye GR Green (Excellgen, Budapest, Hungary), and the banding patterns were visualised under ultraviolet light (254 nm). In case of PCR products assigned to *aflR*, *norA*, and *omtA*, the bands were scored as either present or absent for each *A. flavus* isolate. Partial *CaM* sequences were determined at the LGC Genomics GmbH (Berlin, Germany). Sequence analysis was performed by nucleotide–nucleotide BLAST similarity search at the website of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST>), and sequences were, also, compared with our own sequence database. Species identification was determined from the lowest expected value of the BLAST output (Altschul et al. 1990).

Preparation of Aspergilli section *Flavi* extracts and aflatoxin analysis

Extracts of *A. flavus* isolates ($n = 65$) were prepared and analyzed as described previously (Baranyi et al. 2015). Briefly explained, following a microextraction procedure according to Frisvad et al. (2007), three agar plugs (6 mm in diameter

Table 1 The primers used in PCR

Primer	Sequence (5'–3')	Annealing temperature (°C)	Gene	Reference
Cmd-5	CCGAGTACAAGGARGCCTTC	52–55	<i>CaM</i>	Hong et al. (2005)
Cmd-6	CCGATRGAGGTCCATRACGTGG			
Afl-F	GGGATAGCTGTACGAGTTGTGCCAG	57–59	<i>aflR</i>	Varga et al. (2011)
Afl-R	TGGKGGCCGACTCGAGGAAYGGGT			
Nor-1	ACCGCTACGCCGCACTCTCGGCA	64–66	<i>norA</i>	Criseo et al. (2001); Varga et al. (2011)
Nor-2	GTTGGCCGCCAGCTTCGACACAGC			
Omt-1	GTGGACGGACCTAGTCCGACATCAC	59–61	<i>omtA</i>	Geisen (1996); Varga et al. (2011)
Omt-2	GTCGGCGCCACGCACTGGGTTGGGG			

each) were extracted using a mixture of organic solvents methanol/dichloromethane/ethyl-acetate (1/2/3, v/v/v) supplemented with 1% of formic acid. The extracts were ultrasonicated, and the organic phases were separated, filtered through, and evaporated to dryness under a slight stream of nitrogen. Dry extracts were derivatized with trifluoroacetic acid (Fazekas et al. 2005). HPLC (Shimadzu, Japan) equipped with a fluorescence detector set at an excitation wavelength of 365 nm and an emission wavelength of 430 nm was used for AFB₁ analysis. Separations were achieved on a LiChroCART 250 × 4 mm, 5 µm (Merck, Hungary) column coupled with a LiChrospher 100 RP-18 (Merck, Hungary) guard column and an injection volume of 5 µl. The isocratic mobile phase composition was water/methanol/acetonitrile 65/17.5/17.5 (v/v/v), and the flow rate was maintained at 1 ml/min, while the column temperature was 40 °C. For the quantification of AFB₁, the linear calibration curve was used in a concentration range of 6.25 to 100 ng/ml and the equation of resulted calibration curve was $y = 4056.2x - 7321.2$ ($R^2 = 0.9994$).

Cytotoxicity, genotoxicity, and immunomodulation of AFB₁ vs *Aspergilli* section *Flavi* extracts

Cell cultures

Human lung adenocarcinoma cells A549 and human leukemia monocytes THP-1 (European Collection of Cell Cultures, Salisbury, UK) were grown in 75 cm² flasks in RPMI supplemented with 2 mmol/l glutamine, 10% heat-inactivated FBS, penicillin (100 IU/ml; 1 IU 67.7 µg/ml), and streptomycin (100 µg/ml). Cultures were maintained in a moisturized atmosphere with 5% CO₂ at 37 °C and 95% relative humidity.

Preparation of tested compounds

A stock solution of AFB₁ (0.01 mol/l) was prepared in DMSO/70% ethanol mixture 80/20 v/v. Weighed dried fungal extracts were dissolved in 100% DMSO. The final concentrations of AFB₁ and fungal extracts as well as DMSO/ethanol used in the treatments of cells were obtained by dilution with the culture medium. The highest concentration of fungal

extract applied as treatment was limited by the content of DMSO (up to 1%) that showed no significant effect on cell viability. Mass concentrations of fungal extracts used in MTT assay ranged from 0.05 to 0.6 mg/ml, while the corresponding content of AFB₁ in the extract of toxin-producing *A. flavus* was 0.75 to 4.5 µmol/l. Concentrations of the extracts used in other assays were selected according to the results of MTT and corresponded to subcytotoxic concentrations.

MTT proliferation assay

Viability of A549 cells and macrophage-like THP-1 cells was estimated using MTT assay, as described previously (Jakšić Despot et al. 2016). The differentiation of THP-1 cells into macrophages was performed using PMA (20 nm/well). The A549 (10⁴ cells/well) and macrophage-like THP-1 cells (5 × 10⁴ cells/well) were grown in a 96-well flat-bottom microplate in RPMI 1640 medium supplemented with 10% of FBS. Upon 24 h treatment with AFB₁ (0.1 to 100 µmol/l) or with *Aspergilli* extracts (0.05 to 0.6 mg/ml) of AFB₁-producing and non-producing isolates, the concentration that inhibits growth in 50% of cells (IC₅₀) was determined. Following treatment, the medium was removed and 100 µl of MTT-tetrazolium salt reagent diluted in RPMI 1640 medium without FBS (0.5 mg/ml) was added (V = 100 µl per each well). After 3 h of incubation, the medium was replaced with 100 µl of DMSO to dissolve formazan (product of metabolised MTT reagent), and cells were incubated at room temperature on a rotary shaker for 15 min. The absorbance was measured using a microplate reader (LabSystem iEMS, type 1404) at a wavelength of 540 nm. All tests were performed in five replicates, and results were expressed as percentage of control.

Alkaline comet assay

The extent of primary DNA damage produced after 24 h treatment with AFB₁ and *Aspergilli* extracts was assessed in A549 cells using the alkaline comet assay. A standard protocol, proposed by Singh et al. (1988), with minor modifications was used. Before treatment, A549 cells were seeded in 6-well plates (3 × 10⁵ cells per well) in RPMI 1640 medium

supplemented with 10% of FBS. After 24 h of growth, the cell medium was discarded and cells were treated with AFB₁ (at 1 and 10 µM), *Aspergilli* extracts (at 0.05 and 0.1 mg/ml for non-producing and 0.1 and 0.2 mg/ml for AFB₁-producing isolate). Control cell cultures were grown in parallel (with and without 0.1% DMSO/ethanol) for 24 h. After 24 h of treatment, the cells were washed with 1 ml cold PBS, scraped with rubber, and resuspended in 300 µl of PBS. Aliquots (V = 20 µl) of this suspension were used to prepare slides for the comet assay. Briefly, cell samples were mixed with 100 µl 0.5% LMP agarose (in Ca- and Mg-free PBS) and spread onto fully frosted slides, previously pre-coated with 1% and 0.6% NMP agarose. The microgels were allowed to solidify on ice for 10 min. Then, the slides were subjected to lysis at 4 °C in a buffer containing 2.5 mol/l NaCl, 100 mmol/l Na₂EDTA, 10 mmol/l Tris (pH 10), supplemented with 1% Triton-X 100. The lysis step lasted for 1 h and was followed by denaturation in alkaline buffer (10 mmol/l NaOH, 200 mmol/l Na₂EDTA, pH 13), for 20 min. Electrophoresis lasted for 20 min in the same buffer at 25 V and 300 mA. Microgels were, then, neutralised with three changes of 0.4 mol/l Tris/HCl buffer (pH 7.5), for 5 min each. The slides were kept in a humid atmosphere in a dark box at 4 °C until further analysis. Before image analysis, DNA was stained for 10 min with ethidium bromide (20 µg/ml). Slides were scored using an image analysis system (Comet assay IV, Instem-Perceptive Instruments Ltd., UK) connected to a fluorescence microscope (Olympus, Japan). All of the experiments were performed in duplicate, and, in each experiment, images of 200 randomly selected nucleoids (100 nucleoids from each of the two replicate slides) were measured. Only comets with a defined head were scored. As indicators of DNA damage, tail length (presented in micrometres) and tail intensity (i.e., percentage of DNA in the comet tail) were selected (Collins 2004).

Determination of cytokine levels

To establish cytokine levels after 24 h treatment with AFB₁ and *Aspergilli* extracts, THP-1 cells were selected as an appropriate cell model. Cells were first seeded on 24-well cell culture plates (1 × 10⁶ cells per well) in RPMI 1640 medium supplemented with 10% of FBS and differentiated into macrophages for 24 h with addition of 40 nmol/l LM PMA for 24 h. After that, the medium was discarded, and cells were treated with AFB₁ (at 1 and 10 µmol/l LM) and *Aspergilli* extracts (at 0.05 and 0.1 mg/ml for non-producing and 0.1 and 0.2 mg/ml for AFB₁-producing isolate) for 24 h. Control cell cultures were grown in parallel in RPMI 1640 medium supplemented with 10% of FBS (with and without 0.1% DMSO/ethanol) for 24 h. Following treatment, the cell medium was harvested and frozen at −80 °C until analysis. Concentrations of TNF-α, IL-1β, IL-6, IL-8, and IL-17 were

determined in harvested cell medium by DuoSet ELISA kits (R&D Systems, Minneapolis, USA) according to instructions provided by the manufacturer and as described elsewhere (Hulina et al. 2018). Cytokine concentrations were calculated from measured optical densities determined at 450 nm, using a microplate reader (En Vision® Multilabel Plate Reader, Perkin Elmer) and by standard calibration curves, and results of the three replicates were expressed as percentage of control.

Statistics

Data obtained using the MTT test, comet assay, and cytokine concentration were first evaluated using descriptive statistics. The results were presented as mean ± standard error of mean (SEM). Normality of data distribution was tested by Kolmogorov–Smirnov test; one-way ANOVA was applied for normally distributed data (MTT test and cytokine concentrations) followed by Sidak's post-test, while for non-normally distributed data (comet assay), Kruskal–Wallis test was applied followed by Dunn's multiple-comparison test. A value of $P < 0.05$ was considered statistically significant for all calculations. To obtain the IC₅₀ from the results of the MTT assay, non-linear dose–response fitting was applied using the equation $y = A1 + (A2 - A1) / (1 + 10^{-(\text{LOG}x - x) * p})$.

Results and discussion

Occurrence of aflatoxigenic *Aspergilli* section *Flavi* in air samples

The abundance of airborne *Aspergillus* species section *Flavi* in the occupational environment (GM) in contrast to inhabited urban homes (AP) and urban home basements (BS) as well as outdoor air (ODA) in the six periods of sampling is presented in Table 2. Only in GM were these *Aspergilli* present throughout the whole year, comprising 1–12% of total viable airborne fungi, with mean absolute concentrations approximately 100 to 2000 times higher than in other indoor, as well as outdoor locations. In the GM, the mean absolute concentrations of *Aspergilli* were highest in March and September (about 2000 cfu/m³), with a maximum of 2 × 10⁴ cfu/m³ in September. Looking at the estimated concentrations (probable viable number), the airborne *Aspergilli* section *Flavi* were present at concentrations between 1.7 × 10³ cfu/m³ (January) and 1.3 × 10⁴ cfu/m³ (March) from January to September and dropped below 1000 cfu/m³ only in November. Comparing the results of the present study with our previous reports, the *Aspergilli* section *Flavi* occurred in significantly higher mean absolute concentrations in the GM (405–2035 cfu/m³) than both *Aspergilli* from the subclade *Versicolores* (6.5–44 cfu/m³) and *Aspergilli* section *Nigri* (20–240 cfu/m³) (Jakšić Despot et al. 2016; Jakšić et al. 2018). Mean concentrations

Table 2 Abundance of Aspergilli from the section *Flavi* in indoor and outdoor air for each sampling period

	Grain mill (GM)			Apartment (AP)			Basement (BS)			Outdoor air (ODA)		
	Absolute	Estimated*	Absolute Range	Estimated*	%	cfu/m ³	Mean ± SD	Range	%	cfu/m ³	Mean ± SD	Range
January	290 ± 218	1743 ± 1576	0–600	0–3942	1.06	–	–	–	–	–	–	–
March	2035 ± 2486	13,370 ± 16,330	0–5000	0–32,850	5.57	–	1 ± 4.5	0–20	0.13	–	–	–
May	405 ± 369	2579 ± 2495	0–1000	0–6570	1.51	–	1 ± 4.5	0–20	0.09	–	–	–
July	1335 ± 751	8771 ± 4932	100–3000	657–19,710	3.34	2 ± 6	–	0–20	0.20	–	2 ± 6	0–20
September	1839 ± 4446	8091 ± 29,129	0–20,000	0–131,400	12.85	1 ± 4.5	1 ± 4.5	0–20	0.22	–	–	–
November	150 ± 176	167 ± 199	0–600	0–690	2.39	–	1 ± 4.5	0–20	0.08	–	6 ± 10	0–20

* The probable number of viable particles calculated from Feller's formula ($Pr = N \frac{1}{N} + 1/N - 2 + 1/N - r + 1$), given by the manufacturer (Merck KGaA, Darmstadt, Germany; Pr =probable statistical total; r =number of cfu counted; N =total number of holes in the sampling head)

%, – Mean concentration of Aspergilli section *Flavi* / mean concentration of total airborne fungi × 100. (Mean concentration of total airborne fungi adopted from Jakšić Despot and Šegvić Klarić (2014))

of viable Aspergilli section *Flavi* were also much higher than those reported in poultry farms, soybean and cotton mills, and the food industry ($17\text{--}137.6 \text{ cfu/m}^3$) in India, Italy, and Egypt (Sharma et al. 2010; Abdel Hameed et al. 2012; Cafarchia et al. 2014). Maximum values of statistically estimated concentrations of Aspergilli section *Flavi* obtained in July ($1.9 \times 10^5 \text{ cfu/m}^3$) and ($1.3 \times 10^6 \text{ cfu/m}^3$) were 10–100-fold higher than concentrations of airborne fungi ($> 10^4 \text{ cfu/m}^3$) considered as a health hazard in non-sensitized subjects (Oppliger et al. 2005). Contrary to the GM, Aspergilli section *Flavi* were rarely present or were in low concentrations (maximum 20 cfu/m^3) in urban apartments and basements (AP and BS) and outdoors which is in agreement with recently conducted studies in homes of low-income areas in Syracuse (NY, USA) as well as in homes of hematologic patients in Germany (Crawford et al. 2015; Schweer et al. 2016). In most periods, these Aspergilli comprised between 0.1 and 0.2% of total viable airborne fungi recovered from AP, BS, and ODA.

Based on *CaM* gene region sequencing, among a total of 67 Aspergilli section *Flavi* isolates, 65 were identified as *A. flavus* and 2 as *A. parasiticus* (Table 3) (data on *A. parasiticus* are not shown; results were published in Baranyi et al. 2015). The majority of *A. flavus* strains (89%) were recovered from the GM. Among these strains, 76% produced AFB₁; the highest amounts of AFB₁ ($10\text{--}30 \text{ µg/ml}$) were produced by strains isolated in March and November. For five strains of *A. flavus*, isolated from the GM environment, production of low amounts of AFB₂ ($0.03 \pm 0.02 \text{ µg/ml}$) was detected, in addition to AFB₁ ($13.4 \pm 9.8 \text{ µg/ml}$). These five strains represented 10% of all *A. flavus* isolates from the GM environment. The production of AFB₁ and AFB₂ correlated with genes detected for the transcriptional regulator of aflatoxin biosynthesis (*aflR*), norsolorinic acid reductase (*norA*), and O-methyltransferase gene (*omtA*); 15 strains were positive for two or all three genes for aflatoxin biosynthesis, but the amounts of the toxin were below the LOD. Several earlier studies have suggested the significance of respiratory exposure to *A. flavus* in biowaste-handling facilities (up to 10^5 cfu/m^3) and AFB₁ in grain dust ranging from 0.04 to 107 ng/m^3 (Fischer et al. 2000a,b; Fischer and Dott 2003). Hardin et al. (2009) reviewed the significance of exposure to airborne fungi and mycotoxins in light of concentration of no toxicologic concern-CoNTC (30 ng/m^3), which showed that in occupational environments, such as grain, handling the concentration of AFB₁ in the facilities can exceed the CoNTC. Recently, in poultry farms in Portugal, *A. flavus* was found in three of seven poultry units, and this Aspergilli was the third most frequently found fungal species among indoor airborne fungi (Viegas et al. 2012). In the same study, the presence of aflatoxigenic strains was only confirmed in an outdoor air sample from one of the poultry units but direct evidence of the poultry workers' professional exposure to AFB₁ was found in 58% of positive serum

Table 3 AFB₁-producing abilities of indoor *A. flavus* isolates from different sampling periods and locations

Month of collection	Location	Isolate ID ^a	<i>aflR</i>	<i>norA</i>	<i>omtA</i>	AFB ₁ (ng/ml)
January	GM	AF10527 I.	+	+	+	4.9
		AF10529 I.	+	+	+	4.3
		AF10523 I.	+	+	+	4.1
		AF10525 I.	+	+	+	4.4
March	GM	AF10869B III.	+	+	+	< LOD
		AF10873 III.	+	—	—	< LOD
		AF10868A III.	+	+	+	4.7
		AF10868B III.	+	+	+	12,000.0
May	GM	AF10869A III.	+	+	+	4.8
		AF10923A V.	+	+	+	8.5
		AF10926A V.	+	+	+	5.6
		AF10926B V.	+	+	+	11.0
		AF10923B V.	+	+	+	4.6
		AF10925B V.	+	+	+	11.5
		AF10927A V.	+	+	+	4.3
		AF10930B V.	+	—	—	< LOD
July	BS	AF10959B V.	+	+	+	5.0
	GM	AF10989B VII.	+	+	+	4.6
		AF10992B VII.	+	+	+	4.9
		AF10993A VII.	+	+	+	< LOD
		AF10993B VII.	+	+	+	5.6
		AF10994A VII.	+	+	+	5.0
		AF10995A VII.	+	+	+	4.4
		AF10997A VII.	+	+	+	4.3
		AF10988A VII.	+	+	+	4.9
		AF10988B VII.	+	+	+	4.4
		AF10989A VII.	+	+	+	< LOD
		AF10990B VII.	+	+	+	< LOD
		AF10991A VII.	+	+	+	4.1
		AF10992A VII.	+	+	+	< LOD
		AF10994B VII.	+	+	+	5.0
		AF10996A VII.	+	+	+	204.0
		AF10996B VII.	+	+	+	< LOD
		AF10997B VII.	+	+	+	5.5
		AF11012B VII.	+	+	+	4.6
	AP	AF11008B VII.	+	+	+	< LOD
September	GM	AF11045A IX.	+	+	+	15.6
		AF11046A IX.	+	+	+	< LOD
		AF11048B IX.	+	+	+	4.1
		AF11050A IX.	+	+	+	4.2
		AF11149B IX.	—	—	—	—
		AF11046B IX.	+	+	+	< LOD
		AF11047B IX.	+	+	+	< LOD
		AF11049A IX.	+	+	+	4.71
		AF11049B IX.	+	+	+	657.0
		AF11050B IX.	+	—	+	4.3
		AF11051A IX.	+	+	+	6.2
		AF11053A IX.	+	+	+	5.9
		AF11054A IX.	+	+	+	4.7

Table 3 (continued)

Month of collection	Location	Isolate ID ^a	<i>aflR</i>	<i>norA</i>	<i>omtA</i>	AFB ₁ (ng/ml)
November	GM	AF11110A XI.	+	+	+	11,000.0
		AF11112A XI.	+	+	+	5.0
		AF11113B XI.	+	+	+	4.5
		AF11117B XI.	+	+	+	31,000.0
		AF11118A XI.	+	+	+	4.4
		AF11119A2 XI.	+	+	+	11,000.0
		AF11119B XI.	+	+	+	5.5
		AF11110B XI.	+	+	–	< LOD
		AF11111B XI.	+	–	+	< LOD
		AF11116A XI.	+	+	+	5.0
		AF11119A1 XI.	+	+	+	16,000.0
	BS	AF11144B XI.	+	+	+	< LOD
		AF11140A XI.	+	+	+	5.9
		AF11140B XI.	+	+	+	4.7
	ODA	AF11146B XI.	+	+	+	4.5
		AF11148A XI.	+	+	+	6.1

^a Isolate ID, isolates of *A. flavus* are deposited under their designated ID in the microbial collection (MFBF) at the Department of Microbiology Faculty of Pharmacy and Biochemistry, University of Zagreb

GM, grain mill; AP, apartment; BS, basement; ODA, outdoor air

samples (mean 2 ng/ml) in contrast to the AFB₁-negative serum sampled from control individuals. On the other hand, in living environments, AFB₁ does not seem to be relevant since its formation on building materials during fungal growth has not been detected (Rao 2016; Ren 1999). Our results suggest that a serious threat to human health due to exposure to airborne *A. flavus* could come only in occupational environments, such as grain mill facilities, but not from exposure in urban apartments and basements.

Cytotoxicity of AFB₁ vs *A. flavus* extracts

The cytotoxic potential of *Aspergilli* extracts and AFB₁ applied alone on A549 cells and THP-1 macrophage-like cells is presented in Table 4 and Fig. 1. Although AFB₁ alone significantly reduced the viability of both A549 and THP-1 macrophage-like cells at concentrations up to 100 µmol/l, the reduction did not fall below 50% and the IC₅₀ was not calculated. Our results are consistent with previous studies on A549 cells and human bronchiolar epithelium BEAS-2B cells (Palanee et al. 2001; Vleet et al. 2002; McKean et al. 2006). Van Veelt

et al. (2002) showed that CYP 1A2 and 3A4 enzymes are responsible for the bioactivation of AFB₁ into metabolites (AFB₁ 8,9-epoxide and aflatoxin Q₁) that evoked cytotoxicity in CYP-transfected cells of the bronchiolar epithelium (B-CMV1A2 and B3A4). On the contrary, BEAS-2B and A549 have limited CYP activity, which was in agreement with their CYP gene expression profile (Garcia-Canton et al. 2013) and may explain the weak cytotoxicity of AFB₁ obtained in both A549 and THP-1 macrophage-like cells.

The extract of AFB₁-producing *A. flavus* induced a significant drop in the viability of A549 cells, while the non-producing strain was not cytotoxic to them. THP-1 macrophage-like cells were more sensitive to both extracts, but the AFB₁-producing strain exerted stronger cytotoxicity with IC₅₀ (0.37 ± 0.024 mg/ml; the corresponding AFB₁ concentration is 2.78 ± 0.18 µmol/l AFB₁). Thus, we may suggest that AFB₁ contributed to the *A. flavus* extract cytotoxicity but only in THP-1 macrophage-like cells. Piecková and Wilkins (2004) showed that endo- and exometabolite extracts of dust-borne *A. flavus* were able to stop chicken tracheal cilia beating after the first 24 h of activity. Recently, it was shown that exposure

Table 4 The calculated IC₅₀ of AFB₁ negative and positive *A. flavus* extracts (0.05–0.6 mg/ml) in A549 and THP-1 macrophage-like cell lines using non-linear curve fitting (0.9914)

Treatment	IC ₅₀ (A549) ($\bar{x} \pm \text{SEM}$)	IC ₅₀ (THP-1) ($\bar{x} \pm \text{SEM}$)
<i>A. flavus</i> (AFB ₁ -negative)	> 0.6 mg/ml	> 0.4 mg/ml
<i>A. flavus</i> (AFB ₁ -positive)	> 0.4 mg/ml	0.37 ± 0.024 mg/ml (AFB ₁ 2.78 µM)
AFB ₁	> 100 µM	> 100 µM

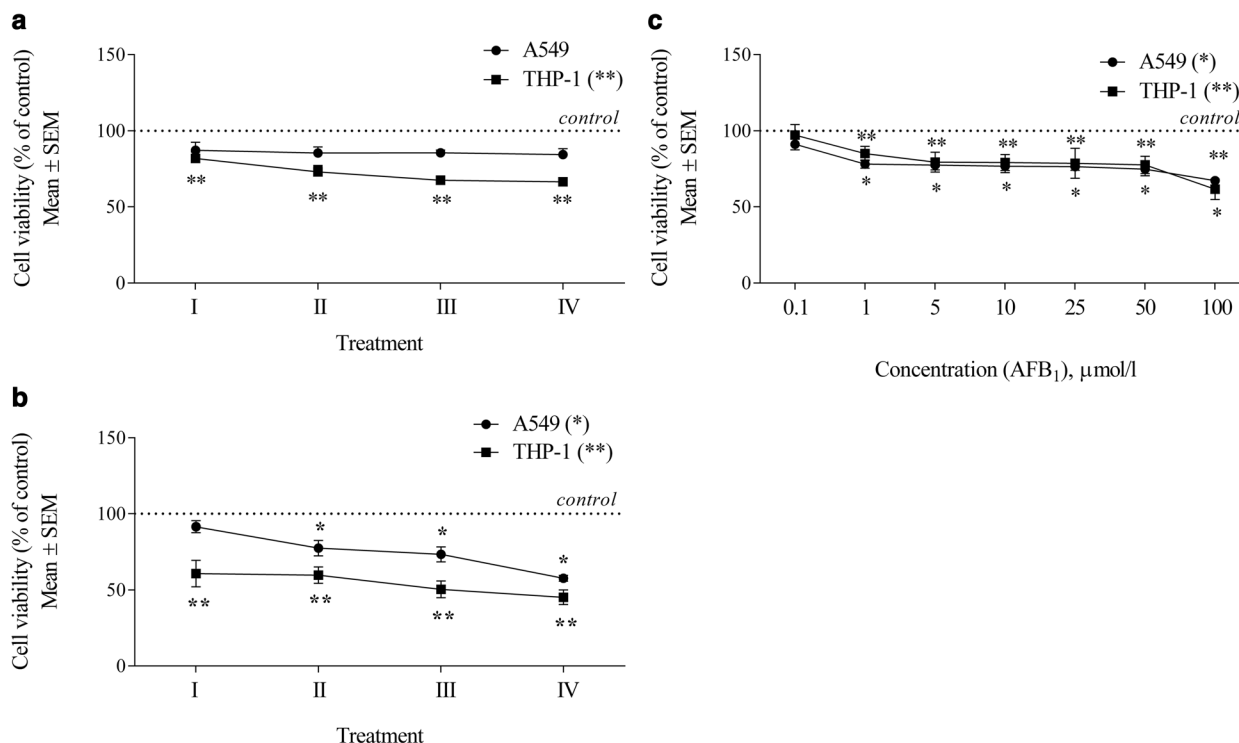


Fig. 1 Survival of THP-1 macrophage-like cells and A549 cells after 24 h of the treatment with the extract prepared from AFB₁-negative (a) and AFB₁-positive (b) isolate of *A. flavus* and single AFB₁ (c). Mass concentrations of fungal extracts prepared from AFB₁-negative isolate of *A. flavus* were I 0.05, II 0.1, III 0.25, and IV 0.4 mg/ml (a), in the extract of toxin-producing *A. flavus* I 0.1, II 0.2, III 0.4, and IV 0.6 mg/ml,

while the corresponding content of AFB₁ was 0.75, 1.5, 3.0, and 4.5 μmol/l, 1–4 respectively (b). Each data point represents the mean ± SEM of cell viability (% of control, control = 100% of cell viability). *, **Significantly different as compared to control in A549 and THP-1 macrophage like cells, respectively ($P < 0.05$)

of primary human sinonasal and bronchial cell cultures to aflatoxins (0.1 to 10 μmol/l) as well as to conditioned media from *A. flavus* reduced the ciliary beat frequency. The effect was blocked by an anti-aflatoxin antibody suggesting that the aflatoxin was responsible for the reduction of ciliary beat frequency (Lee et al. 2016). The same study revealed that AFB₂ activates protein kinase C in A549 cells, which has been linked to inflammation and apoptosis (Diaz-Meco and Moscat 2012; Zhao et al. 2012). Taken together, our results suggest that although AFB₁ alone exerted weak cytotoxic properties in THP-1-like macrophages and A549 lung cells, it significantly contributes to the cytotoxicity of *A. flavus* extracts. Therefore, chronic exposure to aflatoxins in the mixture of *A. flavus* metabolites in an occupational environment may stimulate epithelial damage in airways accompanied by lowered macrophage viability contributing to the pathogenesis of respiratory diseases.

Genotoxicity of AFB₁ vs *A. flavus* extracts

Genotoxic effects were evaluated in A549 cells using the alkaline comet assay (Fig. 2). AFB₁ alone evoked significant concentration-dependent DNA damage measured as tail intensity but not in terms of tail length as compared to control.

Contrary to AFB₁, *Aspergilli* extracts provoked a significant concentration-dependent increase of tail length but not tail intensity. The difference in the responses at DNA level observed after the treatments could be explained by the intrinsic differences in the mechanisms behind DNA lesions. Without conducting additional experiments, we cannot establish the exact mechanism(s) responsible for the observed effects. However, as a comet's tail length is proportional to the number of relaxed DNA loops (Collins et al. 2008), it seems that treatment with *Aspergilli* extracts led to an increased number of relaxed DNA loops, but without a concomitant increase in the amount of DNA breaks. This could be, at least in part, associated with the chemical composition of the extract, which—besides AFB₁—contains other active compounds as well, which possess different potentials to damage DNA or even modulate the DNA damaging effects of AFB₁ itself.

When discussing the significance of the comet assay parameters evaluated in this study, it has to be stressed that tail intensity has been deemed the most useful because as the level of damage increases so does the relative intensity of DNA staining in the tail, rather than tail length (Collins et al. 2008). Therefore, the significantly increased tail intensity we recorded at both of the tested concentrations represents an important piece of information regarding AFB₁ genotoxicity,

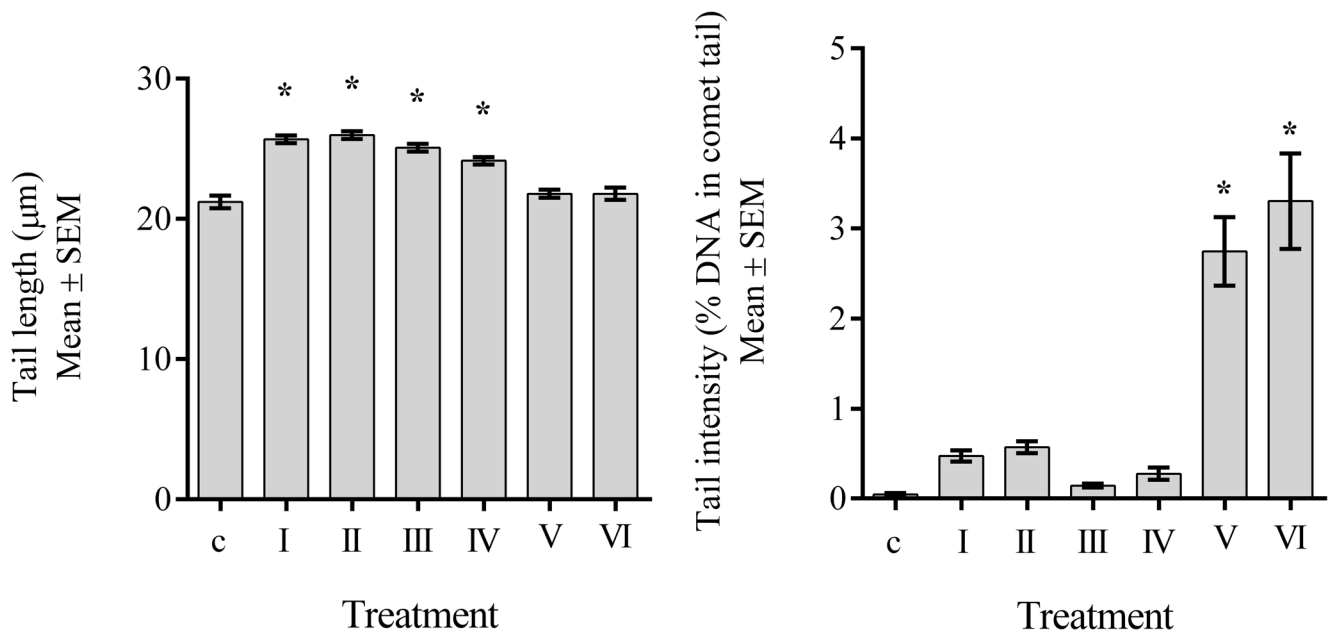


Fig. 2 Genotoxicity of AFB₁-negative and AFB₁-positive extracts of *A. flavus* isolates as well as AFB₁ applied in subcytotoxic concentrations determined by alkaline comet assay as tail length and tail intensity in A549 cells. C control (0.1% DMSO), I AFB₁-negative extract of *A. flavus* 0.05 mg/ml, II AFB₁-negative extract of *A. flavus*

0.1 mg/ml, III AFB₁-positive extract of *A. flavus* 0.1 mg/ml, IV AFB₁-positive extract of *A. flavus* 0.2 mg/ml, V AFB₁ 1 μmol/l, VI AFB₁ 10 μmol/l. The statistical significance of the treatment compared to control is marked with an asterisk (*, $P < 0.05$) above each histogram

which will be useful for planning future experiments with other cell types and exposure scenarios.

The underlying mechanism of AFB₁ genotoxicity was previously studied in the liver in vivo as well as in liver cells and several human bronchial cell lines with good expression of CYP 1A2 and 3A4 enzymes responsible for AFB₁ biotransformation into reactive AFB₁ 8,9-epoxide that binds to DNA (reviewed in Marchese et al. 2018). An association between lung cancer development following AFB₁ exposure was described in workers occupationally exposed to grain dust contaminated with this compound (Hayes et al. 1984). It was demonstrated that AFB₁ exposure induced the production of a DNA binding metabolite (epoxide) by lung cytosols which was correlated with lipoxygenase and prostaglandin H synthase and increased human pulmonary susceptibility to AFB₁ (Massey et al. 2000). Also, oxidative DNA damages in mouse lung cells were correlated to AFB₁ genotoxicity, owing to the induction of 8-hydroxy-20-deoxyguanosine (8-OHdG) formation (Guindon et al. 2008). Although A549 cells possess a limited expression of CYP enzymes (Garcia-Canton et al. 2013), our results suggest that it was sufficient to induce DNA damage upon exposure to low concentrations of AFB₁. In our previous study, AFB₁ (at 5 μmol/l) also evoked significant DNA damage and mutagenic activity, as revealed by the alkaline comet assay, and led to micronuclei formation (Jakšić et al. 2012). Since both the AFB₁-positive and the AFB₁-negative *Aspergilli* extracts exerted similar genotoxic action in A549 cells, we may hypothesize that other metabolites present

in the extract might antagonise AFB₁'s genotoxic action in AFB₁-producing strains.

Immunomodulatory effects of AFB₁ vs *A. flavus* extracts

Many macrophage and epithelial cells produce cytokines and chemokines after challenges from various inflammatory stimuli. Thus, we explored the differences in the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-17) by THP-1 macrophage-like cells upon exposure to single AFB₁ or *Aspergilli* extracts (Fig. 3). TNF-α is an endogenous pyrogen and immunoregulatory cytokine responsible for the production of IL-1, IL-6, and IL-8. Deficiency of TNF-α in experimental animals promoted cancer (Suganuma et al. 1999). TNF-α stimulates polymorphonuclear leukocytes to damage *Aspergillus* hyphae enhancing phagocytosis (Roilides et al. 2002). IL-1β is a potent pro-inflammatory cytokine secreted by macrophages and monocytes which has a stimulatory effect on CD4⁺ T cells promoting differentiation into the T helper cell. It has a beneficial role of mediating an immune response against pathogenic infiltration, but it can also promote the pathogenesis of tissue damage that leads to chronic inflammation (Eder 2009; Turner et al. 2014). Similarly to TNF, IL-6 is a pleiotropic cytokine showing both pro-inflammatory and anti-inflammatory activities, but, in chronic inflammation, it is rather pro-inflammatory. The synthesis of this cytokine is induced by IL-1 and TNF-α (Duque and

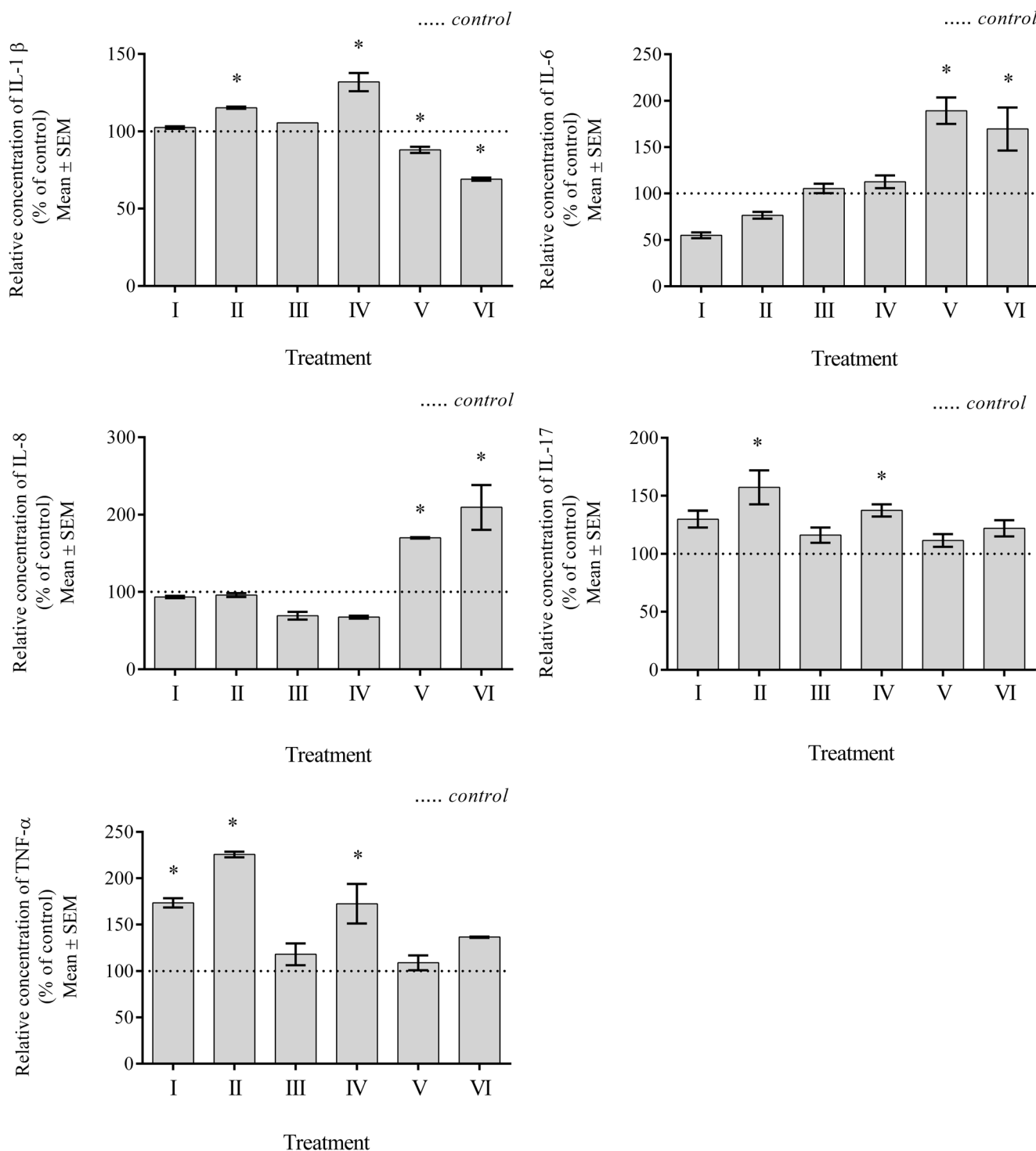


Fig. 3 Relative concentration of cytokines measured in the supernatant of THP-1 macrophage-like cells upon treatment: I AFB₁-negative extract of *A. flavus* 0.05 mg/ml, II AFB₁-negative extract of *A. flavus* 0.1 mg/ml, III AFB₁-positive extract of *A. flavus* 0.1 mg/ml, IV AFB₁-positive extract of

A. flavus 0.2 mg/ml, V AFB₁ 1 μ mol/l, VI AFB₁ 10 μ mol/l. The statistical significance of the treatment compared to control (0.1% DMSO) is marked with an asterisk (*, $P < 0.05$) above each histogram

Descoteaux 2014). IL-8, also known as chemokine CXCL8, is a monocyte- and macrophage-derived cytokine that serves as a chemoattractant of neutrophils to the site of infection or injury and whose secretion from macrophages can be

stimulated with TNF- α , IL-1 β , or a lipopolysaccharide (Carré et al. 1991). IL-17 is a pro-inflammatory cytokine that plays an essential role in the host's defense against microbial infections and is implicated in various inflammatory

conditions, such as autoimmune diseases, metabolic disorders, and cancer (Gu et al. 2013).

AFB₁ alone vs *Aspergilli* extracts showed differences in the immunomodulatory pattern. AFB₁ has the most pronounced concentration-dependent effect on IL-6 and IL-8 excretion. Although the release of TNF- α and IL-17 was also increased by AFB₁ in a concentration-dependent manner, it was not significantly different from controls. Additionally, a difference in IL-1 β release by THP-1 macrophage-like cells upon treatment with single AFB₁ vs *Aspergilli* extract was also observed; AFB₁ decreased IL-1 β levels, while both *Aspergilli* extracts increased the release of this cytokine in a concentration-dependent manner. Several in vivo studies have also shown that AFB₁ stimulates the expression of IL-6 (Hinton et al. 2003; Meissonnier et al. 2008; Qian et al. 2014; Abbès et al. 2016). The same in vivo studies suggested that secretion of TNF- α significantly depends on the administered dose of AFB₁; e.g., a dose of 1.8 mg/kg AFB₁ during four weeks of administration resulted in an increased expression of TNF- α (Meissonnier et al. 2008), while at a significantly lower dose (80 μ g/kg), AFB₁ decreased TNF- α (Abbès et al. 2016). In primary alveolar pig macrophages, AFB₁ (1–100 ng/ml) did not have an effect on the expression of both TNF- α and IL-1 β but decreased phagocytosis efficiency (Liu et al. 2002). The inhibition of macrophage phagocytic activity was also established in vivo in a study on pigs which found that the inhalation of 16.8 μ g/kg of AFB₁ inhibited the phagocytic activity of alveolar macrophages without recovery of function two weeks after inhalation (Jakab et al. 1994). Similarly to our study, AFB₁ evoked an increased expression of IL-8 mRNA in human lymphoblastoid Jurkat T-cells. The activation of an inflammatory response and over-active IL-8-induced recruitment of neutrophils to specific tissues can result in extensive tissue damage (Luongo et al. 2014). As in our study, IL-17 levels increased as the dose increase of AFB₁ in mice liver did, but no differences were detected between the treated and control groups (Ishikawa et al. 2017). Opposite to AFB₁, both *Aspergilli* extracts provoked a significant increase of TNF- α , IL-1 β , and IL-17 in THP-1 macrophage-like cells, while not inducing a release of IL-6 and IL-8. The excretion of TNF- α , IL-1 β , and IL-17 was probably affected by the other metabolites in the extracts of *A. flavus* that could have acted in synergy with AFB₁. At the same time, the metabolite mixtures in the extract could have had an antagonising activity on the induction of IL-6 and IL-8. Additionally, IL-17 appeared to play a central role in eosinophil extravasation from the blood into the lungs of mice upon intranasal exposure to *A. fumigatus* conidia (Murdock et al. 2012), which also contains metabolite mixture. Taken together, AFB₁ elevated IL-6 and IL-8 while *Aspergilli* extracts increased IL-1 β , TNF- α , and IL-17 release in THP-1 macrophage-like cells suggesting that AFB₁ alone and both AFB₁-positive and AFB₁-negative *Aspergilli* extracts could potentially impart adverse effects on

innate immunity but with different mechanisms that could be influenced by the metabolite mixture composition in the extracts.

Considering the limitations of in vitro experiments, we may only suggest that chronic inhalatory exposure to AFB₁ and/or other *A. flavus* metabolites in occupational environments can stimulate epithelial damage of airways accompanied by lowered macrophage viability but with a different underlying mechanism depending on the extrolite profile of the airborne *A. flavus*.

Acknowledgments This work was financially supported by the University of Zagreb (Grant No. 1126). This study forms part of the project GINOP-2.3.2-15-2016-00012, supported by the European Social Fund. This work was also supported by OTKA grant Nos. K115690 and K8407 as well as through András Szekeres, who received support through the new national excellence program of the Hungarian Ministry of Human Capacities.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

- Abbès S, Ben Salah-Abbès J, Jebali R, Younes RB, Oueslati R (2016) Interaction of aflatoxin B₁ and fumonisin B₁ in mice causes immunotoxicity and oxidative stress: possible protective role using lactic acid bacteria. *J Immunotoxicol* 13:46–54. <https://doi.org/10.3109/1547691X.2014.997905>
- Abdel Hameed AA, Ayesha AM, Abdel Razik Mohamed M, Abdel Mawla H (2012) Fungi and some mycotoxins producing species in the air of soybean and cotton mills: a case study. *Atmos Pollut Res* 3: 126–131. <https://doi.org/10.5094/APR.2012.012>
- Altschul S, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Baranyi N, Jakšić Despot D, Palágyi A, Kiss N, Kocsubé S, Szekeres A, Kecskeméti A, Bencsik O, Vágvolgyi C, Šegvić Klarić M, Varga J (2015) Identification of *Aspergillus* species in Central Europe able to produce G-type aflatoxins. *Acta Biol Hung* 66:339–347. <https://doi.org/10.1556/018.66.2015.3.9>
- Bennett JW, Klich M, Mycotoxins M (2003) Mycotoxins. *Clin Microbiol Rev* 16:497–516. <https://doi.org/10.1128/CMR.16.3.497>
- Cafarchia C, Camarda A, Iatta R, Danesi P, Favuzzi V, Di Paola G, Pugliese N, Caroli A, Montagna MT, Otranto D (2014) Environmental contamination by *Aspergillus* spp. in laying hen farms and associated health risks for farm workers. *J Med Microbiol* 63:464–470. <https://doi.org/10.1099/jmm.0.065946-0>
- Campbell-Platt G (1994) Fermented foods—a world perspective. *Food Res Int* 27:253–257. [https://doi.org/10.1016/0963-9969\(94\)90093-0](https://doi.org/10.1016/0963-9969(94)90093-0)
- Carré PC, Mortenson RL, King TE, Noble PW, Sable CL, Riches DW (1991) Increased expression of the interleukin-8 gene by alveolar macrophages in idiopathic pulmonary fibrosis. A potential mechanism for the recruitment and activation of neutrophils in lung fibrosis. *J Clin Invest* 88:1802–1810. <https://doi.org/10.1172/JCI115501>

- Collins AR (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol* 26:249–261. <https://doi.org/10.1385/MB:26:3:249>
- Collins AR, Oscoz AA, Brunborg G, Gaivão I, Giovannelli L, Kruszewski M, Smith CC, Stetina R (2008) The comet assay: topical issues. *Mutagenesis* 23:143–151. <https://doi.org/10.1093/mutage/gem051>
- Crawford JA, Rosenbaum PF, Anagnost SE, Hunt A, Abraham JL (2015) Indicators of airborne fungal concentrations in urban homes: understanding the conditions that affect indoor fungal exposures. *Sci Total Environ* 517:113–124. <https://doi.org/10.1016/j.scitotenv.2015.02.060>
- Criseo G, Bagnara A, Bisignano G (2001) Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group. *Lett Appl Microbiol* 33:291–295
- Diaz-Meco MT, Moscat J (2012) The atypical PKCs in inflammation: NF- κ B and beyond. *Immunol Rev* 246:154–167. <https://doi.org/10.1111/j.1600-065X.2012.01093.x>
- Duque GA, Descoteaux A (2014) Macrophage cytokines: involvement in immunity and infectious diseases. *Front Microbiol* 5:1–12. <https://doi.org/10.3389/fimmu.2014.00491>
- Eder C (2009) Mechanisms of interleukin-1 β release. *Immunobiology* 214:543–553. <https://doi.org/10.1016/j.imbio.2008.11.007>
- Fazekas B, Tar A, Kovács M (2005) Aflatoxin and ochratoxin A content of spices in Hungary. *Food Addit Contam* 22:856–863. <https://doi.org/10.1080/02652030500198027>
- Fischer G, Dott W (2003) Relevance of airborne fungi and their secondary metabolites for environmental, occupational and indoor hygiene. *Arch Microbiol* 179:75–82. <https://doi.org/10.1007/s00203-002-0495-2>
- Fischer G, Müller T, Schwalbe R, Ostrowski R, Dott W (2000a) Exposure to airborne fungi, MVOC and mycotoxins in biowaste-handling facilities. *Int J Hyg Environ Health* 203:97–104. [https://doi.org/10.1078/S1438-4639\(04\)70014-0](https://doi.org/10.1078/S1438-4639(04)70014-0)
- Fischer G, Müller T, Schwalbe R, Ostrowski R, Dott W (2000b) Species-specific profiles of mycotoxins produced in cultures and associated with conidia of airborne fungi derived from biowaste. *Int J Hyg Environ Health* 203:105–116. [https://doi.org/10.1078/S1438-4639\(04\)70015-2](https://doi.org/10.1078/S1438-4639(04)70015-2)
- Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U (2007) Fumonisin B production by *Aspergillus niger*. *J Agric Food Chem* 55(23):9727–9732
- Garcia-Canton C, Minet E, Anadon A, Anadon A, Meredith C (2013) Metabolic characterization of cell systems used in in vitro toxicology testing: lung cell system BEAS-2B as a working example. *Toxicol in Vitro* 27:1719–1727. <https://doi.org/10.1016/j.tiv.2013.05.001>
- Geisen R (1996) Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Syst Appl Microbiol* 19:388–392. [https://doi.org/10.1016/S0723-2020\(96\)80067-1](https://doi.org/10.1016/S0723-2020(96)80067-1)
- Godet M, Munaut F (2010) Molecular strategy for identification in *Aspergillus* section *Flavi*. *FEMS Microbiol Lett* 304:157–168. <https://doi.org/10.1111/j.1574-6968.2009.01890.x>
- Gu C, Wu L, Li X (2013) IL-17 family: cytokines, receptors and signaling. *Cytokine* 64:389–399. <https://doi.org/10.1016/j.asieco.2008.09.006.EAST>
- Guindon KA, Foley JF, Maronpot RR, Massey TE (2008) Failure of catalase to protect against aflatoxin B₁-induced mouse lung tumorigenicity. *Toxicol Appl Pharmacol* 227:179–183. <https://doi.org/10.1016/j.taap.2007.10.015>
- Hardin BD, Robbins CA, Fallah P, Kelman BJ (2009) The concentration of no toxicologic concern (CoNTC) and airborne mycotoxins. *J Toxicol Environ Heal—Part A Curr Issues* 72:585–598. <https://doi.org/10.1080/15287390802706389>
- Hayes RB, van Nieuwenhuize JP, Raatgever JW, ten Kate FJ (1984) Aflatoxin exposures in the industrial setting: an epidemiological study of mortality. *Food Chem Toxicol* 22:39–43. [https://doi.org/10.1016/0278-6915\(84\)90050-4](https://doi.org/10.1016/0278-6915(84)90050-4)
- Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW (2007) *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. *Microbiology* 153:1677–1692. <https://doi.org/10.1099/mic.0.2007/007641-0>
- Hinton DM, Myers MJ, Raybourne RA, Francke-Carroll S, Sotomayor RE, Shaddock J, Warbritton A, Chou MW (2003) Immunotoxicity of aflatoxin B₁ in rats: effects on lymphocytes and the inflammatory response in a chronic intermittent dosing study. *Toxicol Sci* 73:362–377. <https://doi.org/10.1093/toxsci/kfg074>
- Hong S-B, Go S-J, Shin H-D, Frisvad JC, Samson RA (2005) Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* 97:1316–1329. <https://doi.org/10.3852/mycologia.97.6.1316>
- Hulina A, Grdić Rajković M, Jakšić Despot D, Jelić D, Dojder A, Čepelak I, Rumora L (2018) Extracellular Hsp70 induces inflammation and modulates LPS/LTA-stimulated inflammatory response in THP-1 cells. *Cell Stress Chaperones* 23:373–384. <https://doi.org/10.1007/s12192-017-0847-0>
- Ishikawa AT, Hirooka EY, e Silva PLA, Bracarense APFRL, Flaiban KKMD, Akagi CY, Kawamura O, Costa MCD, Itano EN (2017) Impact of a single oral acute dose of aflatoxin B₁ on liver function/cytokines and the lymphoproliferative response in C57BL/6 mice. *Toxins* 9:1–10. doi: <https://doi.org/10.3390/toxins9110374>
- Jakab GJ, Hmieleski RR, Zarba A, Hemenway DR, Groopman JD (1994) Respiratory aflatoxicosis: a suppression of pulmonary and systemic host defenses in rats and mice. *Toxicol Appl Pharmacol* 125:198–205. <https://doi.org/10.1006/TAAP.1994.1065>
- Jakšić Despot D, Šegvić Klarić M (2014) A year-round investigation of indoor airborne fungi in Croatia. *Arh Hig Rada Toksikol* 65:209–218. <https://doi.org/10.2478/10004-1254-65-2014-2483>
- Jakšić Despot D, Kocsubé S, Bencsik O, Kecskeméti A, Szekeres A, Jelić D, Kopjar N, Vágvolgyi C, Varga J, Šegvić Klarić M (2016) Species diversity and cytotoxic potency of airborne sterigmatocystin-producing *Aspergilli* from the section *Versicolores*. *Sci Total Environ* 562:296–304. <https://doi.org/10.1016/j.scitotenv.2016.03.183>
- Jakšić D, Puel O, Canlet C, Kopjar N, Kosalec I, Šegvić Klarić M (2012) Cytotoxicity and genotoxicity of versicolorins and 5-methoxysterigmatocystin in A549 cells. *Arch Toxicol* 86:1583–1591. <https://doi.org/10.1007/s00204-012-0871-x>
- Jakšić D, Kocsubé S, Bencsik O, Kecskeméti A, Szekeres A, Jelić D, Kopjar N, Vágvolgyi C, Varga J, Šegvić Klarić M (2018) Fumonisin production and toxic capacity in airborne black *Aspergilli*. *Toxicol in Vitro* 53:160–171. <https://doi.org/10.1016/j.tiv.2018.08.006>
- Kelly JD, Eaton DL, Guengerich FP, Coulombe RA Jr (1997) Aflatoxin B₁ activation in human lung. *Toxicol Appl Pharmacol* 144:88–95. <https://doi.org/10.1006/taap.1997.8117>
- Lee RJ, Workman AD, Carey RM, B1 C, Rosen PL, Doghramji L, Adappa ND, Palmer JN, Kennedy DW, Cohen NA (2016) Fungal aflatoxins reduce respiratory mucosal ciliary function. *Sci Rep* 6:1–13. <https://doi.org/10.1038/srep33221>
- Liu BH, Yu FY, Chan MH, Yang YL (2002) The effects of mycotoxins, fumonisin B₁ and aflatoxin B₁, on primary swine alveolar macrophages. *Toxicol Appl Pharmacol* 180:197–204. <https://doi.org/10.1006/taap.2002.9406>
- Luongo D, Russo R, Balestrieri A, Marzocco S, Bergamo P, Severino L (2014) In vitro study of AFB₁ and AFM₁ effects on human lymphoblastoid Jurkat T-cell model. *J Immunotoxicol* 11:353–358. <https://doi.org/10.3109/1547691X.2013.848250>
- Manikandan P, Varga J, Kocsubé S, Anita R, Revathi R, Németh TM, Narendran V, Vágvolgyi C, Panneer Selvam K, Shobana CS, Babu Singh YR, Kredics L (2013) Epidemiology of *Aspergillus* keratitis at a tertiary care eye hospital in South India and antifungal

- susceptibilities of the causative agents. *Mycoses* 56:26–33. <https://doi.org/10.1111/j.1439-0507.2012.02194.x>
- Marchese S, Polo A, Ariano A, Velotto S, Costantini S, Severino L (2018) Aflatoxin B₁ and M₁: biological properties and their involvement in cancer development. *Toxins* 10:214. <https://doi.org/10.3390/toxins10060214>
- Massey TE, Smith GBJ, Tam AS (2000) Mechanisms of aflatoxin B₁ lung tumorigenesis. *Exp Lung Res* 26:673–683
- McKean C, Tang L, Tang M, Billam M, Wang Z, Theodorakis CW, Kendall RJ, Wang JS (2006) Comparative acute and combinative toxicity of aflatoxin B₁ and fumonisin B₁ in animals and human cells. *Food Chem Toxicol* 44:868–876. <https://doi.org/10.1016/j.fct.2005.11.011>
- Medina A, Rodriguez A, Magan N (2014) Effect of climate change on *Aspergillus flavus* and aflatoxin B₁ production. *Front Microbiol* 5:348. <https://doi.org/10.3389/fmicb.2014.00348>
- Meissonnier GM, Pinton P, Laffitte J, Cossalter AM, Gong YY, Wild CP, Bertin G, Galtier P, Oswald IP (2008) Immunotoxicity of aflatoxin B₁: impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. *Toxicol Appl Pharmacol* 231:142–149–142–149. <https://doi.org/10.1016/j.taap.2008.04.004>
- Murdock BJ, Falkowski NR, Shreiner AB, Sadighi Akha AA, McDonald RA, White ES, Toews GB, Huffnagle GB (2012) Interleukin-17 drives pulmonary eosinophilia following repeated exposure to *Aspergillus fumigatus* conidia. *Infect Immun* 80:1424–1436. <https://doi.org/10.1128/IAI.05529-11>
- Olsen J, Dragsted L, Autrup H (1988) Cancer risk and occupational exposure to aflatoxins in Denmark. *Br J Cancer* 58:392–396. <https://doi.org/10.1038/bjc.1988.226>
- Oppliger A, Rusca S, Charrière N, Vu Duc T, Droz PO (2005) Assessment of bioaerosols and inhalable dust exposure in Swiss sawmills. *Ann Occup Hyg* 49:385–391. <https://doi.org/10.1093/annhyg/meh105>
- Palanee T, Dutton MF, Chuturgoon AA (2001) Cytotoxicity of aflatoxin B₁ and its chemically synthesised epoxide derivative on the A549 human epithelioid lung cell line. *Mycopathologia* 151:155–159. <https://doi.org/10.1023/A:1017985924257>
- Pariza MW, Johnson EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Regul Toxicol Pharmacol* 33:173–186. <https://doi.org/10.1006/rtp.2001.1466>
- Paterson RRM, Lima N (2010) How will climate change affect mycotoxins in food? *Food Res Int* 43:1902–1914. <https://doi.org/10.1016/j.foodres.2009.07.010>
- Perrone G, Gallo A, Logrieco AF (2014) Biodiversity of *Aspergillus* section *Flavi* in Europe in relation to the management of aflatoxin risk. *Front Microbiol* 5:1–5. <https://doi.org/10.3389/fmicb.2014.00377>
- Piecková E, Wilkins K (2004) Airway toxicity of house dust and its fungal composition. *Ann Agric Environ Med* 11:67–73
- Qian G, Tang L, Guo X, Wang F, Massey ME, Su J, Guo TL, Williams JH, Phillips TD, Wang JS (2014) Aflatoxin B₁ modulates the expression of phenotypic markers and cytokines by splenic lymphocytes of male F344 rats. *J Appl Toxicol* 34(3):241–249. <https://doi.org/10.1002/jat.2866>
- Rao CY, Fink RC, Wolfe LB, Liberman DF, Burge HA (2016) A study of Aflatoxin production by growing on wallboard. *Journal of the American Biological Safety Association* 2(4):36–42
- Ren P, Ahearn DG, Crow Jr SA (1999) Comparative study of *Aspergillus* mycotoxin production on enriched media and construction material. *J Ind Microbiol Biotechnol* 23(3):209–213
- Roilides E, Lammaigne CG, Farmaki E (2002) Cytokines in immunodeficient fungal infections: an emerging patients therapy. *Int J Infect Dis* 6:154–163
- Samson RA, Houbaken J, Thrane U, Frisvad JC, Andersen B (2010) Food and indoor fungi. Fungal Biodiversity Centre Utrecht, The Netherlands
- Samson RA, Visagie CM, Houbaken J, Hong S-B, Hubka V, Klaassen CHW, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsubé S, Szigeti G, Yaguchi T, Frisvad JC (2014) Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud Mycol* 78:141–173
- Schweer KE, Jakob B, H2 C, Fischer G, Vehreschild M, Cornely OA, Vehreschild JJ (2016) Domestic mould exposure and invasive aspergillosis—air sampling of *Aspergillus* spp. spores in homes of hematological patients, a pilot study. *Med Mycol* 54:576–583. <https://doi.org/10.1093/mmy/myw007>
- Sharma D, Dutta BK, Singh AB (2010) Exposure to indoor fungi in different working environments: a comparative study. *Aerobiologia (Bologna)* 26:327–337. <https://doi.org/10.1007/s10453-010-9168-9>
- Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175:184–191
- Soares C, Rodrigues P, Peterson SW, Lima N, Venâncio A (2012) Three new species of *Aspergillus* section *Flavi* isolated from almonds and maize in Portugal. *Mycologia* 104:682–697. <https://doi.org/10.3852/11-088>
- Suganuma M, Okabe S, Marino MW, Sakai A, Sueoka E, Fujiki H (1999) Essential role of tumor necrosis factor α (TNF- α) in tumor promotion as revealed by TNF- α deficient mice. *Cancer Res* 59:4516–4518
- Turner MD, Nedjai B, Hurst T, Pennington DJ (2014) Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta—Mol Cell Res* 1843:2563–2582. <https://doi.org/10.1016/j.bbamcr.2014.05.014>
- Van Vleet TR, Klein PJ, Coulombe RA (2002) Metabolism and cytotoxicity of aflatoxin B₁ in cytochrome P-450 expressin human lung cells. *J Toxicol Environ Health A* 65:853–867. <https://doi.org/10.1080/0098410029007121>
- Varga J, Frisvad JC, Samson RA (2011) Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Stud Mycol* 69:57–80. <https://doi.org/10.3114/sim.2011.69.05>
- Viegas S, Veiga L, Malta-Vacas J, Sabino R, Figueredo P, Almeida A, Viegas C, Carolino E (2012) Occupational exposure to aflatoxin (AFB₁) in poultry production. *J Toxicol Environ Health A* 75:1330–1340. <https://doi.org/10.1080/15287394.2012.721164>
- Yu J, Bhatnagar D, Cleveland TE (2004) Completed sequence of a aflatoxin pathway gene cluster in *Aspergillus parasiticus*. *FEBS Lett* 564:126–130. [https://doi.org/10.1016/S0014-5793\(04\)00327-8](https://doi.org/10.1016/S0014-5793(04)00327-8)
- Zhao M, Xia L, Chen GQ (2012) Protein kinase c delta in apoptosis: a brief overview. *Arch Immunol Ther Exp* 60:361–372. <https://doi.org/10.1007/s00005-012-0188-8>